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Characterization of the *Salmonella paratyphi* C Vi Polysaccharide

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The Vi capsular polysaccharide (Vi) is both a virulence factor and a protective antigen of *Salmonella typhi*; its pathogenic role for *Salmonella paratyphi* C is less well understood. We found no differences between the antigenic and immunogenic properties and the structure of the Vi from representative strains of *S. paratyphi* C, *S. typhi*, and *Citrobacter freundii*. There were, however, differences in both the amount produced per cell and the degree of association with the cell among the Vi from the three species of *Enterobacteriaceae*. *S. paratyphi* C produced less Vi than both the wild-type *S. typhi* and *C. freundii* did, and it showed the fastest release of Vi into the media. These findings may provide an explanation for the inability of the Vi to inhibit completely the agglutination of *S. paratyphi* C by anti-O sera. In an outbreak of enteric fever caused by *S. paratyphi* C, 66 of 78 isolates (85%) were Vi positive.

The Vi capsular polysaccharide (Vi) is a component of several members of the family *Enterobacteriaceae*, including *Salmonella typhi*, *Salmonella paratyphi* C, and *Citrobacter freundii* (4, 11–13, 22, 23, 33). Vi, purified from either *S. typhi* or *C. freundii*, was shown to be a 1→4-linked linear homopolymer of α-D-(1→4)-GalNAcA variably O-acetylated at the C3 position (16). The virulence-promoting and protective properties of the Vi for *S. typhi* have been established by the following data: (i) *S. typhi* isolates from the blood of patients with typhoid fever are invariably Vi positive (Vi^+), whereas strains isolated from the stools or urine may be Vi^+ or Vi negative (Vi^-) (6, 11–13, 17, 18, 22); (ii) Vi^+ strains have a lower 50% lethal dose than Vi^- strains for mice (11–13, 17, 18); (iii) volunteers challenged with Vi^+ strains of *S. typhi* had a higher incidence of disease, including bacteremia and fever, than those challenged with Vi^- strains (17, 18); and (iv) two controlled clinical trials, in areas with high attack rates of typhoid fever, established the protective action of a Vi vaccine prepared under nondegrading conditions (1, 25).

C. freundii is rarely a cause of invasive disease in humans and is usually found in clinical specimens as an opportunistic pathogen (32). There is controversy regarding this organism as a causative agent of diarrhea (9). In an unpublished study, we could not find Vi^+ strains among *C. freundii* isolates from patients (J. B. Robbins and R. Schneerson).

Little is known about the pathogenesis of invasive infections caused by *S. paratyphi* C. Unlike *S. typhi*, *S. paratyphi* C is pathogenic for animals as well as for humans (13, 22, 23, 28). This pathogen is only a minor cause of enteric fevers in South America, Asia, and the United States. However, in certain parts of Africa, occasional outbreaks of enteric fever caused by *S. paratyphi* C have been reported (14, 19). The frequency of Vi^+ strains among clinical isolates of *S. paratyphi* C and their relation to the severity of the disease caused by this organism are unknown. By using agglutination reactions with rabbit typing antisera, Kauffmann demonstrated serological identity between the Vi from *S. typhi* and *S. paratyphi* C (23). Although the Vi is a surface structure of *S. paratyphi* C, it did not exert the same degree of inhibition upon the agglutination of this organism by anti-O antibodies as was observed with *S. typhi*; i.e., the presence of the Vi reduced but did not eliminate the agglutinability of Vi^+ strains of *S. paratyphi* C in anti-O sera (13, 22, 23). Kauffmann also noted that reversion of Vi^+ to Vi^- strains (VW variation) occurred at a higher frequency in *S. paratyphi* C than in *S. typhi* (23, 24).

Kauffmann argued that the Vi was not a virulence factor because its occurrence on *S. paratyphi* C was not related to an increase in lethality of this pathogen in mice (22–24). Later, Mandel et al., by using a single-parent *S. paratyphi* C, showed that Vi^+ organisms had a lower 50% lethal dose in mice than did Vi^- organisms. Actively induced or passively administered Vi antibodies stimulated by either *S. typhi* or *S. paratyphi* C did not confer protection from lethal challenge of mice by Vi^+ or Vi^- strains of *S. paratyphi* C in this model. These same antibodies protected mice challenged with a Vi^+ strain of *S. typhi* (28). The structure of the Vi from *S. paratyphi* C has not been reported.

We report studies on the structure, immunological properties, and rate of synthesis of the Vi of *S. paratyphi* C compared with *S. typhi* and *C. freundii*. The occurrence of the Vi on *S. paratyphi* C strains isolated from patients during an outbreak of enteric fever is also reported.

MATERIALS AND METHODS

Bacteria. Strains of *S. typhi*, *S. paratyphi* C, and *C. freundii* used for growth, immunologic, and kinetics studies are listed in Table 1. Keith Klugman and Hendrik Kornhof, Department of Microbiology, South African Institute for Medical Research, Johannesburg, Republic of South Africa, collected 78 isolates of *S. paratyphi* C from patients hospitalized with enteric fever during an epidemic caused by this pathogen during 1985 to 1987. The strains were stored freeze-dried until use. Each strain was reconstituted with sterile saline, streaked onto tryptic soy broth (Difco Laboratories, Detroit, Mich.) in 0.9% agarose (FMC Corp., Marine Colloids Div., Rockland, Maine) and incubated at

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TABLE 1. Bacterial strains

Strain designation	Source
S. paratyphi C 09322	Human blood isolate, 1987; Kathmandu, Nepal
S. paratyphi C 11494	Human blood isolate, 1986; Eastern Transvaal, RSA[a]
S. paratyphi C 12786	Human blood isolate, 1986; Eastern Transvaal, RSA
S. paratyphi C 33593	Human blood isolate, 1987; Eastern Transvaal, RSA
S. paratyphi C 128600	Human blood isolate, 1987; Eastern Transvaal, RSA
S. typhi B1	Human blood isolate, 1986; Kathmandu, Nepal
S. typhi B8	Human blood isolate, 1986; Kathmandu, Nepal
S. typhi S3	Human blood isolate, 1986; Kathmandu, Nepal
S. typhi Ty2	Food and Drug Administration
C. freundii WR7011	Louis Baron, Walter Reed Army Institute of Research

[a] RSA, Republic of South Africa.

37°C overnight. Individual colonies were streaked onto antiserum agar (see below).

Immunology. Antisera were raised in rabbits by multiple intravenous injections of Formalin-inactivated bacteria as previously described (2). The strains used were *S. paratyphi* C 09322 and *C. freundii* WR7011. Rabbit *S. typhi* Ty2 antiserum containing 550 μg of Vi antibody per ml was generously supplied by Louis Baron, Walter Reed Army Institute of Research, Washington, D.C. Hyperimmune burro antiserum (B 260), elicited by *S. typhi* Ty2, has been described (29). Double immunodiffusion and rocket immunoelectrophoresis (RIE) were performed as previously described (39). Vi antibodies were measured by radioimmunoassay (29).

Antiserum agar. Plates were prepared as previously described (29) by using B 260 antiserum to a final concentration of 8.0%. Bacteria were streaked out; the plates were incubated at 37°C overnight, and they were inspected for halos surrounding individual colonies.

Preparation of *S. paratyphi* C Vi. *S. paratyphi* C 128600 was cultivated in a 50-liter fermentor containing 40 liters of 0.1 M sodium phosphate, pH 7.4, 5 liters of 10% D-glucose, and 5 liters of 10% yeast extract (Difco) dialysate (C. Rubinowitz and J. Shiloach, Biotechnology Unit, National Institute of Diabetes and Digestive and Kidney Diseases). The organisms were cultivated with high aeration and vigorous stirring to the end of the log phase of growth. Formalin was then added to the fermentor to a final concentration of 0.5%, and the temperature was lowered to 10°C. The bacterial suspension was centrifuged after 7 h, at a time that no viable bacteria could be detected. The supernatant fluid was added to an equal volume of 0.2% cetavlon (Sigma Chemical Co., St. Louis, Mo.). The suspension was thoroughly stirred, left overnight at 3 to 8°C, and centrifuged. The pellet was then extracted sequentially with H_2O, 0.1 M NaCl, and 1.0 M NaCl. The supernatant from the 1.0 M NaCl extraction was brought to 70% ethanol and left overnight at 3 to 8°C. The precipitate was collected by centrifugation, washed with 99% ethanol, and air dried. The resultant powder was treated with DNase, RNase, and proteinase (Sigma) (21), extracted with cold phenol, and then ultracentrifuged at 35,000 rpm (Beckman 28-55M ultracentrifuge) at 5°C for 5 h. The final product (1.6 mg per liter of culture) had less than 1% protein and nucleic acids (38, 40). The molecular sizes of three preparations were similar, exhibiting a broad peak on gel filtration through C1-2B Sepharose, with a partition coefficient of about 0.3 (40).

Synthesis and cell association of Vi. Bacteria were cultivated at 37°C with aeration and vigorous stirring in 50 ml of trypic soy broth. The inocula were 20 μl of bacteria from an overnight culture. The pH and optical density at 560 nm were recorded on samples removed at 4, 7, 10, and 24 h. The Vi in the supernatant and on the twice-washed cells was measured by RIE by using Vi from *S. typhi* Ty2 as a reference standard and B 260 antiserum.

^{13}C NMR. As described for the analysis of the Vi from *S. typhi* and *C. freundii* (37), both the native and alkali-treated spectra of *S. paratyphi* C were obtained. The *S. paratyphi* C Vi preparation was treated with 0.1 M NaOH at 37°C for 12 h, neutralized with HCl, and dialyzed against deionized water for 2 days at 3 to 8°C. The sample was centrifuged at 40,000 rpm (Beckman 65 ultracentrifuge) at 10°C for 5 h, and the ^{13}C nuclear magnetic resonance (NMR) spectrum of the supernatant was analyzed (8).

Calorimetric reactions. The metachromatic reaction product between the Vi from *C. freundii*, *S. typhi*, or *S. paratyphi* C and the cationic dye, acridine orange, was performed as described by Stone and Szu (36). The percentage of *O*-acetyl groups on the native Vi was determined as described by Hestrin (15).

RESULTS

Halo formation on antiserum agar. All Vi^+ strains of *S. typhi*, *S. paratyphi* C, and *C. freundii* WR7011 formed halos of precipitation that were immunologically specific (halos were not observed with *H. influenzae* type b or group C meningococcal antiserum plates). There were differences observed among the halos formed by the three organisms. In general, halos formed by *S. paratyphi* C were less dense than those formed by *S. typhi* and *C. freundii* (Fig. 1).

Immunogenicity of Vi^+, non-*S. typhi* strains. Preimmunization sera from B 260, as well as from rabbits, had low or nondetectable levels of Vi antibodies. Table 2 shows that multiple intravenous injections with either *C. freundii* WR7011 or *S. paratyphi* C 09322 elicited high levels of Vi antibodies which declined in 2 months. Reinjection of these bacteria elicited an increase in Vi antibodies; only *C. freundii* WR7011 elicited a booster response.

Immunodiffusion analyses. A reaction of identity was observed among the Vi preparations from *S. typhi* Ty2, *S. paratyphi* C 09322, and *C. freundii* WR7011 when reacted against B 260 (Fig. 2A). Identical results were obtained with rabbit antisera elicited by either *C. freundii* WR7011, *S. paratyphi* C 09322, or *S. typhi* Ty2 (data not shown). Figure 2B shows the reaction of identity obtained by reacting these rabbit antisera against the Vi from *S. paratyphi* C. An additional line of precipitation adjacent to the antigen well was formed between this Vi and the *S. paratyphi* C antiserum. This line was likely due to lipopolysaccharide-antibody interaction and was not observed with the *S. typhi* antiserum because of the differences in group C and group D O-specific side chains of these *Salmonella* lipopolysaccharides (27). All rabbit antisera also showed a reaction of identity with the Vi from *S. typhi* and *C. freundii* (data not shown).

Synthesis and cell association of Vi on representative strains of *S. paratyphi* C, *S. typhi*, and *C. freundii*. The kinetics of synthesis and release of Vi into the culture supernatant were studied in *S. paratyphi* C 09322 and 128600, *S. typhi* B8 and Ty2, and *C. freundii* WR7011. The highest levels in both the culture supernatant and cell-associated Vi were produced by

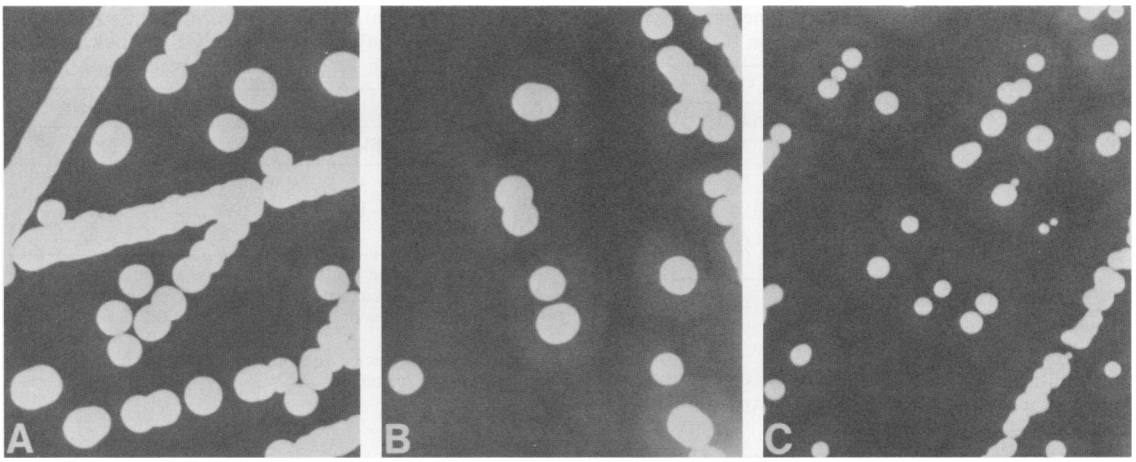

FIG. 1. Specific immunoprecipitin halos on B 260 antiserum agar surrounding Vi^+ colonies of *S. paratyphi* C 128600 (A), *S. typhi* B8 (B), and *C. freundii* WR7011 (C).

C. freundii (21 μg/ml). *S. typhi* B8 synthesized more Vi (17 μg/ml) than did *S. paratyphi* C 128600 (12 μg Vi per ml). *S. typhi* Ty2, maintained in the laboratory for many years, produced 7 μg of Vi per ml. *S. paratyphi* C 09322 lost its ability to produce halos in antiserum agar after two laboratory passages and produced very little Vi (1 μg/ml). Similar patterns of production and release of Vi were found when three other *S. paratyphi* C strains (11494, 12786, and 33593) and two other *S. typhi* strains (B1 and S3) were compared (data not shown).

Table 3 shows both the amount of Vi released into the media and the Vi that was cell associated after 24 h. Only *C. freundii* retained a significant amount of cell-associated Vi (15%). The strains of *S. typhi* and *S. paratyphi* C released greater than 98% of their Vi into the media. The rate of release into the media was faster in *S. paratyphi* C than in *S. typhi* or *C. freundii*.

Structure of *S. paratyphi* C Vi by ^{13}C NMR. The ^{13}C NMR spectrum of the native Vi of *S. paratyphi* C was similar to that of *C. freundii* and *S. typhi* in that it displayed relatively sharp and resolved resonances for the corresponding methyl carbon atoms of the *N*-acetyl groups (CH_3 resonances at ca. 20 to 25 ppm and C(O) resonances at ca. 177 ppm). The ring carbon atoms showed a broad, unresolved band, probably as a result of decreased mobility of these residues relative to the acetyls (data not shown). From these methyl resonances, we would conclude that the majority of the C-2 and C-3 hydroxyls were acetylated to approximately the same extent, since the ratio of the intensities of the *N*- and *O*-acetyl resonances was about 1:1. This was found in all Vi antigens. Thus, the native Vi antigens showed a similar behavior in their NMR spectra, including the extent of O acetylation. By using Hestrin's method (15), the native Vi of *S. paratyphi* C was determined to be 65% O acetylated. Sharper NMR spectra were obtained from the Vi from *C. freundii* and *S. typhi* after each had been treated with alkali to remove the *O*-acetyl groups. The major resonances for the NaOH-treated Vi of *S. paratyphi* C corresponded to those of base-treated Vi from *C. freundii* and *S. typhi* (Table 4); it may be concluded that the Vi from *S. paratyphi* C is an O-acetylated α-(1→4)-linked linear homopolymer of GalNAcA. Since it was necessary to remove the *O*-acetyl groups to sharpen the ^{13}C NMR spectrum, we are unable to observe the site of acetylation. However, the structure permits only C-3 to be available for acetylation (C-6 is not available for O acetylation because it is a carboxyl group, not hydroxymethyl). In addition, the metachromatic reaction product between the Vi from *C. freundii*, *S. typhi*, or *S. paratyphi* C and the cationic dye acridine orange exhibited the same degree of spectral shift and hypochromism (data

TABLE 2. Serum Vi antibodies in rabbits injected intravenously with *S. paratyphi* C 09322 or *C. freundii* WR7011

Rabbit	Immunizing bacteria	μg of antibodies/ml at days post immunization:						
		0	35	50	75[a]	95	100	115
ED4	*S. paratyphi* C	0.02	875	650	80	510	380	190
ED5	*S. paratyphi* C	0.04	310	115	65	NA[b]		
ED6	*S. paratyphi* C	0.06	405	185	40	155	115	70
ED1	*C. freundii*	0.02	185	79	11	500	230	155
ED2	*C. freundii*	0.21	205	105	11	525	435	156
ED3	*C. freundii*	0.06	405	185	40	155	115	70

[a] Pre-second immunization.
[b] NA, Not available, rabbit died.

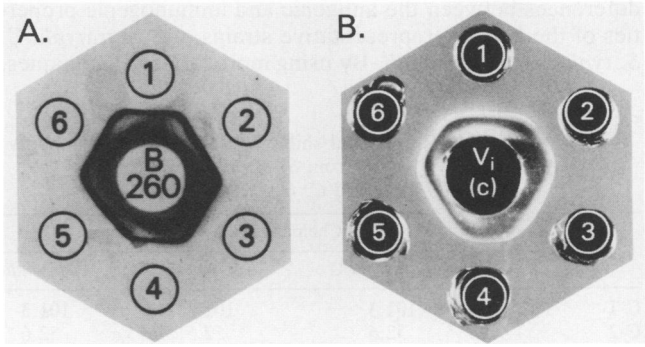

FIG. 2. Double immunodiffusion pattern of *S. typhi* Ty2 antiserum with the Vi from various bacteria (A). Center well, B 260 antiserum; outer wells 1 and 4, *C. freundii* WR7011; well 2, *S. paratyphi* C 09322; wells 3 and 6, *S. typhi* Ty2; and well 5, *S. paratyphi* C 128600. Double immunodiffusion pattern of *S. paratyphi* C with various antisera (B). Center well, Vi from *S. paratyphi* C 09322; outer wells contain hyperimmune rabbit antisera elicited by *S. paratyphi* C 09322 (wells 1 and 5), *C. freundii* WR7011 (wells 2 and 4), and *S. typhi* Ty2 (wells 3 and 6).

TABLE 3. Amount of Vi released into the media and cell associated after 24 h of cultivation

Bacterial strain	μg of Vi/ml at OD_{560}[a]		
	Supernatant	Cell associated	Combined
S. paratyphi C 128600	12.0	0.25	12.3
S. paratyphi C 09322	1.07	0.01	1.08
S. typhi B8	17.3	0.01	17.3
S. typhi Ty2	7.33	0.01	7.34
C. freundii WR7011	18.7	2.47	21.1

[a] OD_{560}, Optical density at 560 nm.

not shown). This similarity of metachromatic reaction provides evidence that the negative charge distribution of the three Vi preparations are similar (36).

Prevalence of Vi among strains of *S. paratyphi* C from the eastern Transvaal, Republic of South Africa. A total of 78 clinical isolates were analyzed. The ages were obtained for 52 of the patients: 47 were ≤13 years old, the youngest was 1 month, and the oldest was 36 years old. The origins of 70 of 78 of these samples was known: 53 were from blood, 10 were from stool, and 7 were from urine. Most of the strains (51 of 78 or 65%) were Vi^+ by the antiserum agar technique (Table 5). Two of the Vi^+ strains underwent VW form variation (24), and eight others lost their ability to produce halos after two to three laboratory passages. The Vi^+ strains were found in 37 of 53 isolates from the blood, 6 of 10 from the stool, 5 of 7 from the urine, and 3 of 8 from isolates for which the source was unstated. The remaining 27 isolates that were negative for Vi by the antiserum agar technique were examined further by RIE. Small rockets of precipitation were obtained for 15 of these strains, indicating that some of the colonies had Vi^+ organisms. With the data obtained by the two techniques, 66 of 78 (85%) of the *S. paratyphi* C isolates from this outbreak were Vi^+.

DISCUSSION

There has been controversy about the occurrence and the role of the Vi in virulence and protective immunity of *S. paratyphi* C (3, 4, 7, 11–13, 22, 23, 26, 28). Earlier workers were unable to isolate the Vi from *S. paratyphi* C in pure form. Its structure has not been reported. We found no differences between the antigenic and immunogenic properties of the Vi from representative strains of *S. paratyphi* C, *S. typhi*, and *C. freundii*. By using more modern techniques,

TABLE 4. ^{13}C NMR chemical shifts[a] and tentative assignments of O-deacetylated Vi from *S. paratyphi* C 128600, *S. typhi* Ty2, and *C. freundii* WR7011

Atom	Chemical shifts in ppm for:		
	S. paratyphi C	*S. typhi*	*C. freundii*
C-1	101.3	101.3	101.3
C-2	52.6	52.5	52.6
C-3	70.0	70.4	69.8
C-4	80.8	80.8	81.1
C-5	74.6	74.1	74.6
C-6	177.6	176.3	177.0
C(O)-CH_3	25.3	25.3	25.3
C(O)-CH_3	177.6	177.7	177.7

[a] Chemical shifts are referenced to sodium 3-(trimetylsilyl) propanoate 2,2,3,3-d4 as an internal reference; spectra were recorded at approximately 60 to 70°C as D_2O solutions at pH 7.

TABLE 5. Vi[a] on strains of *S. paratyphi* C isolated from patients during an outbreak (1985 to 1987) of enteric fever in the eastern Transvaal, Republic of South Africa

Vi status	No. of isolates from:			
	Blood	Stool	Urine	Unstated
Vi^+	32	4	4	3
Vi^-	16	4	2	5
Mixed[b]	5	2	1	0

[a] Vi was detected by halo formation in antiserum agar as previously described (20). The concentration of B 260 hyperimmune antiserum, prepared by multiple injections of *S. typhi* Ty2, was 8.0% in tryptic soy broth in 0.9% agarose.

[b] Halos were detected surrounding most colonies, but there were at least two halo-negative colonies.

we confirmed that alkali-treated Vi from *S. paratyphi* C, *S. typhi*, and *C. freundii* are identical. Vi from *S. typhi* and *C. freundii* are variably O acetylated at the C-2 position from 59 to 90% (Szu, S.C. and X. Li, unpublished data). We also found that native Vi from *S. paratyphi* C was O acetylated to the same extent at 65%.

Comparison of Vi content among Vi organisms relied on indirect methods, such as mouse potency (26) and adsorption techniques (10). By using RIE for measurements of cell association and release of Vi, we found differences both in the total amount of Vi synthesized and the amount released from the cell surface among the three bacterial species. *C. freundii* WR7011 synthesized the highest amount and had the most cell-associated Vi of the three species. This strain, which was selected for its high capsule content, could serve as a source for vaccine production. Wild-type *S. typhi* synthesized higher amounts of Vi than did *S. paratyphi* C. *S. typhi* Ty2, a laboratory-passaged organism for about 60 years, synthesized less Vi than one *S. paratyphi* C strain recently isolated from the blood of patients. Kauffmann reported that strains of *S. paratyphi* C rapidly lost their Vi expression when passed on laboratory media (24). We confirmed this report, since *S. paratyphi* C 09322 lost its Vi expression after two passages in the laboratory. *S. paratyphi* C showed the fastest release of Vi into the media. The findings of lower production and faster release into the media provide an explanation for the inability of the Vi to inhibit completely the agglutination of *S. paratyphi* C by anti-O sera.

The rate of synthesis and cell association properties of the Vi may be genetically controlled. The genetic basis of Vi antigen expression involves both a structural determinant (via B) and a functional determinant (via A), which occupy identical chromosomal sites in *S. typhi* and *S. paratyphi* C (20, 34). Variable expression of the Vi in *C. freundii* WR7004 has been shown to be due to an invertible insertion sequence located with the *C. freundii* via B genes (30, 35). Similarly, rapid loss of Vi expression by *S. paratyphi* C Vi may involve special mechanisms within either of the two chromosomal determinants.

The Vi of *S. typhi* (group D) enhances the virulence of the organism and enables it to survive in the bloodstream (31); Vi^- strains of this pathogen, as well as nontyphoidal strains of group D salmonellae, are still capable of causing enteric fever (5). Expression of the Vi also enhanced the virulence of *S. paratyphi* C in mice (17, 18); its role in human disease has not been determined. Outbreaks of enteric fever caused by *S. paratyphi* C have been reported in Africa (19). There have been no reports, however, of the Vi status of strains

isolated during an outbreak of enteric fever caused by *S. paratyphi* C. Antiserum agar and RIE analyses of strains isolated from patients during an outbreak in South Africa showed that 66 of 78 (85%) of the *S. paratyphi* C strains were Vi^+. Our findings that (i) 2 Vi^+ strains showed VW form variation; (ii) 8 Vi^+ strains lost their ability to produce halos after two to three laboratory passages; and (iii) 15 of the strains that were halo negative were Vi^+ by RIE provide support for Kauffmann's claim and suggest that most *S. paratyphi* C have Vi in the host. A search for the Vi on *S. paratyphi* C freshly isolated from patients could provide further information on this subject.

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